

# DNA Technology, Interstate Commerce, and the Likely Origin of Formosan Subterranean Termite (Isoptera: Rhinotermitidae) Infestation in Atlanta, Georgia

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**ABSTRACT** This study was the first to use DNA sequencing data to infer the origin of a Formosan subterranean termite, *Coptotermes formosanus* Shiraki, infestation. Cytochrome oxidase II gene sequence was used to define eight specific maternal lineages from 14 geographic locations across the southeastern United States, Hawaii, and China. One maternal lineage, A, was unique to all the Atlanta collections and one New Orleans collection, which indicated that termites at these sites shared a common maternal ancestor. The impact of interstate commerce in terms of Formosan subterranean termite introductions is addressed as is the industry response needed to arrest the spread of these termites via commercial traffic in used railroad cross ties.

**KEY WORDS** Formosan subterranean termite, *Coptotermes formosanus*, mtDNA, COII gene

FORMOSAN SUBTERRANEAN TERMITE, *Coptotermes formosanus* Shiraki, infestations were verified in the Atlanta, GA, metropolitan area in 1993 by B. T. Forschler. Seven infestations were confirmed by the end of 1997 with the help of the Georgia Department of Agriculture and local pest control operators. Termite infested landscape timbers (i.e., railroad cross ties removed from grade) were common to all sites. It therefore appears likely that these railroad cross ties harbored the insects before their arrival into Atlanta. Interviews with property owners revealed that the cross ties at several sites were purchased on or between 1989 and 1990. The Georgia State Department of Agriculture located the vendor who sold the cross ties. This vendor, however, could not confirm from whom he purchased the ties, but thought they came in from Charleston, SC. Thus, the geographic origin of the cross ties was unconfirmed.

Mitochondrial DNA (mtDNA) sequence markers, defined as maternal lineages, have been used to track gene flow in termites (Jenkins et al. 1998, 1999, 2001) because the mitochondrial genome is maternally inherited (MacRae and Anderson 1990, Vogler and DeSalle 1993, Jenkins et al. 1996) in insects. Thus, if all or part of a Formosan subterranean termite population was transported from one geographic site to another, it could be traced through these maternal lineages. Furthermore, the cytochrome oxidase gene is the most

widely used mitochondrial protein-coding gene in insects (Simon et al. 1994). There are also numerous published conserved primers (Liu and Beckenbach 1992, Simon et al. 1994) for this gene. It was reasoned, therefore, that the cytochrome oxidase nucleotide data should be useful at the level of Formosan subterranean termite populations. The mitochondrial cytochrome oxidase II gene sequence, therefore, was used in this study to track the origin of the Atlanta Formosan subterranean termite infestation.

The culpability of interstate commerce in the spread of Formosan subterranean termites has been documented (Chambers et al. 1988, Jones and Howell 2000, Woodson et al. 2001), as has their ability to infest aging railroad ties (Spink 1967, Sponsler et al. 1988). Thus, the threat of *C. formosanus* extending their range throughout the continental United States via interstate commerce in railroad ties and/or other wood products is significant. The specific implications of the mitochondrial sequence data; therefore, in terms of population expansion and adaptation as well as the industry response to these DNA results are discussed.

## Materials and Methods

**Clarification of Terms.** Population is defined genetically as a group of individuals that mate and produce viable offspring. A lineage is defined as a group of consensus cytochrome oxidase sequences—individual sequences that are exactly alike.

**Termite Samples.** All *C. formosanus* specimens were preserved in 70–100% ethyl alcohol. Each individual Atlanta specimen (worker termites only) was ran-

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Table 1. Collection information for each site including the population symbols used to represent these sites

Collection site symbol	# workers sequenced <sup>a</sup>	Collection locations	Collection dates
		Georgia	
GA1	2	Lawrenceville (Gwinnett County)	02/6/99
GA2	5	Tucker (DeKalb County)	14/6/93
GA3	6	Dallas (Paulding County)	06/7/97
GA4	5	Suwanee (Gwinnett County)	09/10/96
		Louisiana	
LA1	5	New Orleans (Chatham Street)	08/2/96
LA2	3	New Orleans (West Bank)	15/5/99
LA3	2	New Orleans (French Quarter)	20/4/94
LA4	2	New Orleans (French Quarter)	08/8/97
		South Carolina	
SC	11	Charleston (Rutledge and Grove St.)	23/2/99
		Alabama	
AL	8	Mobile	05/3/99
		Texas	
TX	3	Ft. Worth	09/6/95
		Hawaii	
HI	2	Honolulu	24/8/94
		China	
CH1	2	Hsin-hui, Kuang-chou Province	1963
CH2	2	Hsin-hui, Kuang-chou Province	1963

<sup>a</sup> Fifty-eight workers (forward and reverse sequences total 116).

domly chosen from sampling localities (Table 1). DNA extraction and sequencing was according to Jenkins et al. (1998). All sampling in Georgia was from homes located in the Atlanta Metropolitan Statistical Area (Table 1; Figs. 1 and 2). *Coptotermes formosanus* samples from China were originally collected in 1963 in Hsin-hui, Kuang-chou Province. Ivan Hrdy at the Institute of Organic Chemistry, Zbraslav, Czech Republic, has maintained inbred colonies from these collections. Randomly selected workers from two of

these colonies, CH1 and CH2 (Table 1), were used in this study for comparison purposes.

**Amplification and Sequencing.** DNA from individual termites was amplified and sequenced. DNA templates for polymerase chain reaction (PCR) amplification consisted of total nucleic acids isolated from individual whole worker termites. DNA extraction was accomplished as described in Liu and Beckenbach (1992) and Jenkins et al. (1999). Oligonucleotide primers TL2-J-3037 (alias A-tLeu) and TK-N-3785 (alias B-tLys) (Liu and Beckenbach 1992, Simon et al. 1994) were used to amplify as well as to prime the sequencing reactions for both strands of a 684-bp fragment of the COII gene. Insect mtDNA was confirmed as described by Jenkins et al. (1998).

The cytochrome oxidase gene was sequenced from randomly selected individual *C. formosanus* collected from 14 geographic locations, 11 in the southeastern United States, one in Hawaii, and two in southeastern China. Although multiple individuals were sequenced from each collection (Table 1), only one genotype was observed per collection. These multiple identical se-

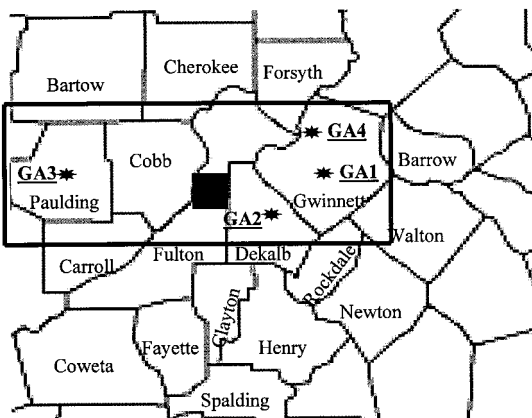


Fig. 1. Sites in the Atlanta area from which collections were made. Atlanta is indicated in black and is composed of central Fulton County and the city of Decatur in DeKalb county. The Atlanta Metropolitan region is composed of >3 million people and encompasses 10 counties: Cherokee, Cobb, Gwinnett, DeKalb, Douglas, Fulton, Clayton, Henry, Fayette, and Rockdale. The Atlanta Metropolitan Statistical Area, however, encompasses >8 million people and is made up of 20 counties that include the 10 above and Paulding, Coweta, and Forsyth among others.

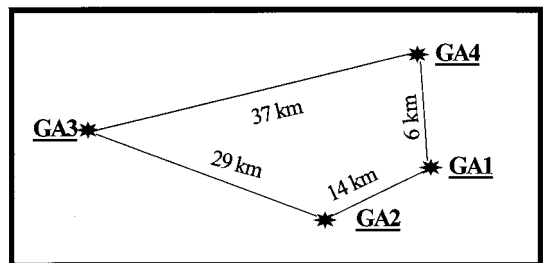


Fig. 2. An enhanced diagram of the collection sites shown in Fig. 1. Driving distances between these sites are designated in kilometers (<http://maps.excite.com>).

**Table 2. Maternal lineages based on COII sequence data and representing maternal ancestry and gene flow**

COII lineages <sup>a</sup>	Population symbols <sup>b</sup>
A	GA1
	GA2
	GA3
	GA4
B	LA1
	LA2
	LA3
	LA4
C	SC
D	AL
E	TX
F	HI
G	CH1
H	CH2

<sup>a</sup> Lineages represent consensus sequence data—all sequences in a lineage were the same.

<sup>b</sup> Refer to Table 1 for information on each collection.

quences were combined into consensus sequences, each of which represented a mitochondrial lineage.

PCR amplification was performed in a standard 25- $\mu$ l reaction (Jenkins et al. 1999). Amplification was accomplished in a Perkin-Elmer Gene Amp PCR system 9600 (PE Applied Biosystems, Foster City, CA) according to Jenkins et al. (1999). Fragments were treated with exonuclease I (10 U/ $\mu$ l) and shrimp alkaline phosphatase (1 U/ $\mu$ l) to remove primers and inactivate dNTPs left over from the PCR reaction, respectively (Jenkins et al. 1998). The DNA (10–20 ng/100 bp PCR product) sequencing reaction was performed with the Dye-Terminator Cycle Sequencing Kit (Perkin-Elmer) in a GenAmp 9600 PCR system. Electrophoresis was then accomplished on a 6% polyacrylamide gel. Reactions were fractionated and sequencing carried out by the ABI 373 automated DNA sequencer system (PE Applied Biosystems, Foster City, CA).

**Sequence Data Analysis.** Sequencer 3.0 software (Gene Codes, Ann Arbor, MI) was used to edit individual ABI chromatograms. Because sequencing was in both directions, contigs were made using the same software. If any sequences were the same they were combined into a single consensus sequence. Multiple consensus sequences of all samples were aligned with Sequencer 3.0 and MALIGN (Hein 1989). Consensus sequences were assigned a unique maternal lineage using designations A–H. Extant population relationships were then determined through a neighbor-joining (Saitou and Nei 1987) analysis using PHYLIP (Phylogeny Inference Package) version 3.5c (Felsenstein 1993). *Coptotermes* sp. (Genbank accession number AB005583) was used to root the tree and thereby establish character state polarities (Avisé 1994).

## Results

*Coptotermes formosanus* consensus sequences and lineages were assigned (Table 2) and extant relationships were determined (Fig. 3) based on the identification of consistently repeatable nucleotide poly-

morphisms or variations that were population-specific (Fig. 4). The consensus sequence polymorphisms were unique for specific collections as well as consistently unique across several collections (Fig. 4). Each unique sequence, therefore, represents a population-specific female lineage (Table 2). Six population-specific lineages (A, B, C, D, E, F) were defined from the United States and two population-specific lineages from China (G, H) (Table 2; Fig. 3).

All Atlanta collections, GA1, GA2, GA3, GA4, and a single New Orleans collection, LA1, (Table 2) had the same sequence and were, therefore, combined into a single consensus sequence defined as lineage A (Table 2; Figs. 3 and 4). Three New Orleans collections, LA2, LA3, LA4 (Table 2), had the same sequence defined by lineage B (Fig. 3). The Charleston, SC, samples defined lineage C; Mobile, AL, lineage D; Honolulu, HI, lineage E; and Ft. Worth, TX, lineage F (Table 2). Among all of the specific lineages there was a total of 15 variable sites (Fig. 4). Two sites, 19 and 661 (Fig. 4), were unique to lineage A, which had six polymorphic sites. Lineages F and E demonstrated four and three polymorphic sites, respectively. Two of these sites, 222 and 297, were shared among lineages E, F, G, and H (Fig. 4). Lineage D had a unique polymorphic site at 333 (Fig. 4). None of the polymorphic sites in lineage C were unique (Fig. 4). This lineage did not fall into a clade or group as the other lineages did (Fig. 3). Lineage H had nine polymorphic sites, four of which, 32, 33, 46, and 211, were unique (Fig. 4). Lineage G had seven polymorphic sites, two of which, 8 and 11, were unique (Fig. 4).

There was no agreement between the Atlanta consensus nucleotide sequence (lineage A) and the Charleston consensus nucleotide sequence (lineage C) (Fig. 4), contrary to the historical evidence obtained from the vendor who thought the cross ties might have been trucked in from Charleston. The gene tree (Fig. 3) resulting from the neighbor-joining (Saitou and Nei 1987) analysis (Felsenstein 1993) grouped Chinese lineages G and H with lineages E (Texas) and F (Hawaii), but differentiated these lineages from all other lineages (Fig. 3).

## Discussion

DNA nucleotide data were used to trace the possible origin of a termite infestation. Our study highlights that these techniques, when used in conjunction with traditional or historic sources, can provide insight into population structure and information on the dispersal of an introduced termite pest. These data have importance, therefore, for Formosan subterranean termite population structure, genetic variation, and interstate commerce as it relates to passive termite dispersal.

**Mitochondrial Sequence Data and Allozyme Data Differentiate Groups.** Allozyme electrophoresis research done by Wang and Grace (2000) differentiated Formosan subterranean infestations in the United States into two distinct groups: Hawaii and Lake Charles, LA, with China in one group and New Or-

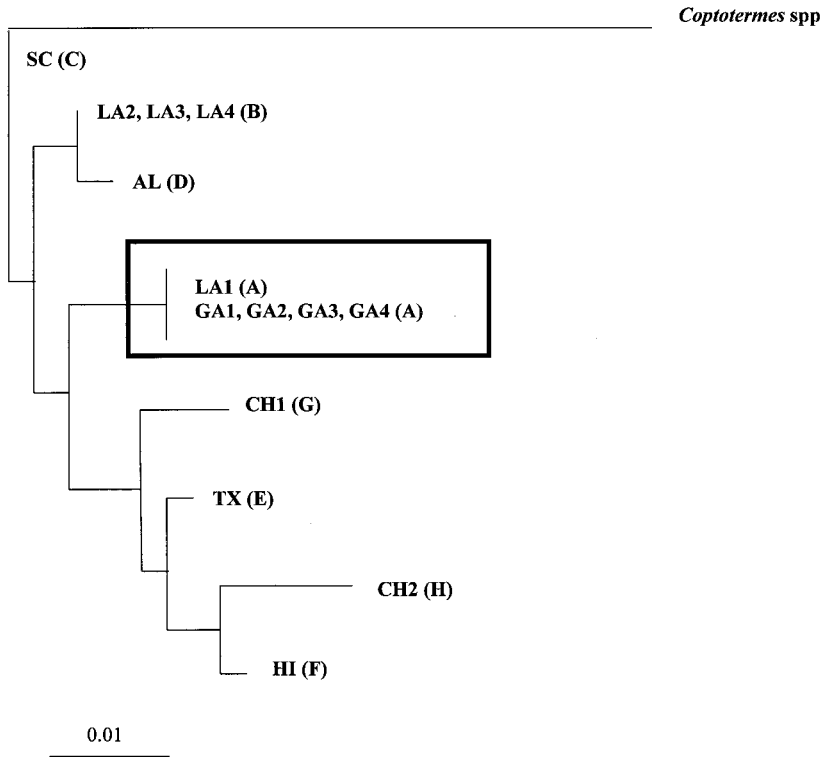


Fig. 3. Neighbor-joining tree (Saitou and Nei 1987) created with PHYLIP 3.5c (Felsenstein 1993) demonstrates relationships among the different female lineages. *Coptotermes* spp. COII sequence, Genbank accession number AB005583, was used to root the tree and against which all specimens were compared. Refer to Table 1 for definition of population symbols and Table 2 for definition of lineages. Lineage A, which encompasses both New Orleans and Atlanta samples, is outlined.

leans and Florida in the other. In the current study, phylogenetic analysis of mitochondrial consensus sequences separated lineages A and B from the China and Hawaii collections (Fig. 3). Lineage A, which comprises all the Atlanta samples and New Orleans LA1 formed a unique clade (Fig. 3) as did lineage B. Lineages F, G, and H also formed a separate clade. Thus, like the Wang and Grace (2000) study, we observed the New Orleans lineages separating from the Hawaii and China lineages. The Atlanta termites, which share a maternal ancestor with the New Orleans LA1 population (Table 2; Fig. 3), may also share with New Orleans populations an introduction history separate from that of these Hawaiian and Chinese termites.

**Atlanta and New Orleans Share Maternal Lineage.** The Atlanta samples shared a common female ancestor with the New Orleans population, LA1 (Table 2; Fig. 3). That this ancestor also can be found in Charleston cannot be ruled out without more exhaustive sampling. New Orleans, however, is a parsimonious choice as the source considering the shared lineage. Also, there is interstate access from New Orleans across the states of Mississippi and Alabama into Atlanta; cross ties are trucked into Atlanta (Forschler et al. 2000).

**Maternal Lineages and Genetic Variation Among Sampled Populations.** Formosan subterranean termite populations form aerial nests (Tamashiro et al. 1987) and may have multiple queens and kings (King and Spink 1969, Wang and Grace 1999), a gene pool composed of millions of termites (Su and Scheffrahn 1990), and a foraging range in excess of 100 linear meters between known feeding sites (King and Spink 1969; Lai 1977). Henderson (1996) also suggested that Formosan colonies could fuse into larger groups to better exploit food resources. Based on all this information, we were surprised to observe only one maternal lineage, A, over all the Atlanta collections. Population fragmentation and founding scenarios could, however, account for this observation.

The large Formosan subterranean termite populations are subject to fragmentation in the process of passive dispersal that does not appear to impact the fragments' propensity for growth and expansion. If a peripheral portion of the population's gene pool is dispersed and if populations maintain maternal kin-biased groups (Jenkins et al. 1999, 2001), then population fragments reasonably represent a fraction of the gene pool that may reflect the narrowly defined genetic lineages observed in this research. A recent

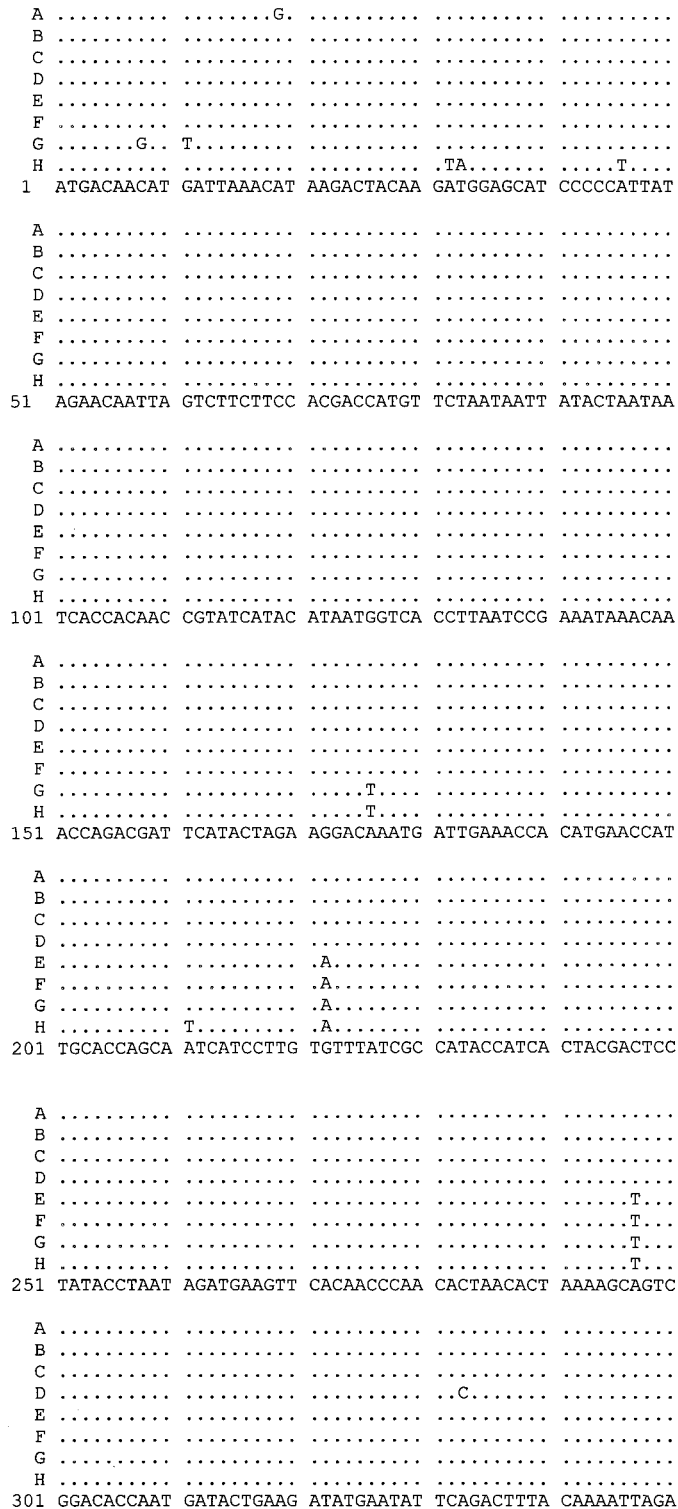


Fig. 4. A total of 685 bases was analyzed. The consensus sequence for each population represented by lineages A, B, C, D, E, F, G, H (Refer to Table 2 for lineage information), identified at the left of each sequence, were aligned for comparison. Out of 685 bases, 15 polymorphic sites are identified among these lineages. The general consensus sequence is defined at the bottom of the alignment.

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A .....
B .....
C .....
D .....
E .....
F .....
G .....
H .....
351 ATTCGACTCA TACATAATCC CCCAAGAAGA ACAACAAGAA AGAACATTC

A .....
B .....
C .....
D .....
E .....
F .....
G .....
H .....
401 GACTACTAGA CACGGACAAC CGAATCGTCC TACCAATCAA TTCACCAATT

A .....
B .....
C .....
D .....
E .....
F .....
G .....
H .....
451 CGACTAATCG TTACAGCAGC AGACGTCCTA CACTCATGAA CAATTCCAAG

A .....
B .....
C .....
D .....
E .....
F .....
G .....
H .....
501 ACTAGGGGTA AAAACAGACG CCACACCAGG ACGACTAAAT CAAACAAGAT

A .....
B .....
C .....
D .....
E .....
F .....
G .....
H .....
551 TCTCAATCAG TCGTCCTGGT ATCCTATATG GACAATGCTC AGAGATTGTC

A .....
B .....
C .....
D .....
E .....
F .....
G .....
H .....
601 GGAGCAAACC ACAGATTCAT ACCCATCACA ATTGAAAGAG TACCAGCAAA

A ..... C..... ..T..... ..G.
B .....
C ..... ..G.
D .....
E .....
F .A..... ..G.
G .A.....
H ..... ..T..... ..G.
651 ATACTTTAT AACTGAGTT CAAACTAAG AGAAT

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Figure 4. Continued

study of the indigenous subterranean termite genus *Reticulitermes* generated the unique hypothesis that these insects might form meta-colonies of kin-biased maternal groups that colonize separate food resources (Jenkins et al. 1999). Thus, the lack of variation in the matrilineal lines from the Atlanta collections could reflect the act in which either a peripheral gene pool, or progeny of a more fecund female representing an expanded area within the population, was splintered off and a maternal kin-biased group tied to New Orleans was transported to Atlanta.

Fragmented Formosan subterranean termite populations or founding populations were likely introduced into the continental United States >50 yr ago (Spink 1967, Weesner 1970, Su and Tamashiro 1987). Infestations, which likely represent only a portion of the original populations, are now firmly established in at least eight states (Woodson et al. 2001). Through stochastic lineage sorting (Avise 1994), the process of differential female fecundity, mortality and mating, maternal lines in these infestations, like the Atlanta infestation, would have been further culled. Furthermore, the phenomenon of reduced variation among Formosan subterranean termite mtDNA has been observed.

Broughton and Grace (1994) examined Formosan mtDNA restriction fragment-length polymorphisms (RFLP) for 10 Hawaiian colonies. They were attempting to establish a colony-specific DNA marker. There was not enough variation among mtDNA haplotypes, however, to establish individual differences much less colony differences (Broughton and Grace 1994). RFLP variation is dependent on mutational changes or nucleotide differences at specific endonuclease target sites. Thus, as Broughton and Grace (1994) suggest, their result may reflect a founding event by few maternal lineages, which resulted in reduced genetic variation.

Genetic variation is important because it impacts on a species' ability to respond to natural selection: selection is inversely proportional to genetic variation (Fisher 1958). This means that genetic structure among populations can limit the response to natural selection within populations. In this study, interdemec genetic structure of the cytochrome oxidase gene in the Formosan populations is small. Because mtDNA has an effective population size one-fourth that of nuclear DNA (Birkey et al. 1983), maternal mitochondrial nucleotide variation of these founding populations would be even more severely affected. This lack of variability observed in the Atlanta populations is consistent with a founding population of relatively recent origin, which is significant information in light of how well the populations appear to be adapting across metropolitan Atlanta. To date, five Metropolitan Atlanta counties, which are crisscrossed by major interstate arteries, have Formosan infestations. These counties include Gwinnett, DeKalb, Fayette, Cobb, and Paulding (Fig. 1).

**Industry Response.** Unwitting movement of termite-infested materials resulting in the establishment of exotic populations is a documented phenomenon in

the entomological literature (Gay 1969, Su and Tamashiro 1987). Subterranean termites have been introduced to nonendemic areas in the southeastern United States (Appel and Sponsler 1989) and elsewhere (Jenkins et al. 2001). Esenther (1961) first associated this movement along railroad lines in Wisconsin, USA. The introduction of termites into Toronto and Winnipeg, Canada was noted in the 1930s and 1970s, respectively (Grace 1989). We also have used three molecular markers to track an infestation of subterranean termites in the United Kingdom that was believed to have arrived via infested wine crates from either southern France or northern Spain (Jenkins et al. 2001).

Infested wood and wood by-products can be transported from one location to another because termites do not often reveal their presence with clear signs of activity. Furthermore, any cellulose source in contact with the ground can become infested with subterranean termites. It is important to recognize, therefore, the potential role that railroad cross ties play in the unwitting movement of exotic termites to new areas.

The Railway Tie Association, as a result of this work (Forschler et al. 2000), has taken the lead in alerting the railroad community to the possible problem of passive Formosan subterranean termite dispersion in railroad cross ties. They drafted a letter with accompanying documents in October of 2000 to the major railroad companies operating in Louisiana. The letter outlined the potential role cross ties removed from grade could play in the spread of the Formosan subterranean termite, and it called for a proactive program to prevent passive introgression into new geographic areas. In response, Norfolk and Southern Corporation issued a directive in December 2000 that essentially placed a moratorium on the use of released railroad cross ties from Louisiana. The directive also called for an industry effort because of the widespread distribution of the Formosan subterranean termite. RailWorks Wood Waste Energy formed a task force in April 2001 to consider development of an efficacious and cost effective industry standard Quality Assurance Program for all areas with Formosan subterranean termite infestations.

We contend that a properly designed Quality Assurance Program must be applicable to all areas of the United States with established Formosan infestations. It should provide a decision matrix for identifying infested cross ties and/or protocols for dealing with released ties. Approaches should form a continuum from a complete moratorium on the use of released cross ties in certain areas, to on-site identification of infested ties and proper disposal. Input should include information from entomologists, the railroads, and the wood preservative community.

The work described in this manuscript demonstrates the need for measures that prevent the transport and establishment of this insect pest as well as the applicability of direct DNA sequencing for tracing the origin of infestations. Information on Formosan subterranean termite gene flow will help to facilitate development of novel treatment strategies and a bet-

ter understanding of the social and ecological considerations affecting range expansion.

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